PURIFICATION OF ERYTHROPOIETIN BY ION-EXCHANGE CHROMOTOGRAPHY

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RANDOLPH AIR FORCE BASE, TEXAS

DECEMBER 1958

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PURIFICATION OF ERYTHROPOIETIN BY ION-EXCHANGE CHROMATOGRAPHY

A method is given for the purification of erythropoietin from the filtrate of acidified, boiled plasma prepared from phenylhydrazine anemic rabbits utilizing DEAE-cellulose ion-exchange columns. The active erythropoietic factor thus prepared has been partially characterized and shown to be an acidic glycoprotein of low molecular weight.

In previous publications (1, 2, 3) we have reported that the erythropoietic activity of acidified, boiled plasma filtrates (APF) prepared from phenylhydrazine anemic rabbits was associated with a protein having the paper electrophoretic mobility of an alpha globulin and the chemical characteristics of an acid glycoprotein. Acidic glycoproteins of small molecular weight are found in Cohn Fraction VI (4). In preliminary work with plasma from phenylhydrazine anemic rabbits and from a patient with polycythemia vera we have found erythropoietic activity in Fraction VI (5). The present study was therefore designed to isolate, by ion-exchange chromatography, acidic glycoproteins from the crude source of boiled anemic rabbit plasma.

MATERIALS AND METHODS

Acidified, boiled plasma filtrate obtained from phenylhydrazine anemic, adult, white male rabbits was prepared by a modification of the method of Gordon (6) as previously described (3). The filtrate was dialyzed in the cold for 48 hours against repeated changes of distilled water and then lyophilized. One liter of plasma yielded 500 to 600 mg. of lyophilizate. The lyophilizate, first being shown to possess erythropoietic activity by our previously described method (3), was fractionated on diethylaminoethyl (DEAE) cellulose ion-exchange columns (7). Two separation procedures were utilized. Procedure II was designed for a more definitive fractionation on the basis of the results obtained with procedure I. The separation experiments are summarized in the elution diagrams in figures 1 and 2. Fraction pools as designated in figure 1 were dialyzed, lyophilized, and redissolved in 0.9 percent NaCl. Along with unmodified APF starting material and control saline injections, these fractions were assayed for erythropoietic activity. Fraction pools as designated in figure 2 were assayed directly, using a solution of 0.9 percent NaCl in 0.01 molar sodium acetate buffer as a control injection. Female Sprague-Dawley rats (average weight, 220 gm.) were injected with the respective fractions from procedure I. The injections were given subcutaneously each day for 4 days with 2 mg. by dry weight (in 2 ml. of 0.9 percent NaCl). The fractions from procedure II were administered on a similar schedule, the daily dose being 0.5 mg. by protein content. The protein content was estimated on the effluent fraction pools by the ratio of optical density measurements at 280 and 260 mμ, according to the method of Warburg and Christian (3). On day 4, the animals were given an intravenous injection of 0.5 μc. of iron-59 as iron citrate contained in 0.25 ml. of solution. The percent of the injected dose of iron-59 appearing in the total red cell mass in 24 hours was estimated by standard scintillation counting technics on whole blood obtained by aortic exsanguination with the animal under ether anesthesia. The total red cell mass was calculated from the microhematocrit assuming an average blood volume of 5.18 ml. per 100 gm.

Received for publication on 28 July 1958.

of body weight. Prior to sacrifice, blood was obtained from the tail vein for reticulocyte enumeration by the dry cresyl-blue method.

RESULTS AND DISCUSSION

The erythropoietic activity of the isolated fractions is presented in tables I and II. Fraction A, washed from the column in the solvent buffer, and fraction B showed no erythropoietic activity. The small peak appearing in the elution diagram before fraction A was also inactive, but was tested in only two animals. Fraction C contained the majority of the erythropoietic activity and was more potent than the starting material. In 0.15 molar NaCl the peak of fraction C was homogeneous in the ultracentrifuge. The centrifuge data suggested a molecular weight of about 10,000. By free electrophoresis, barbiturate buffer pH 8.6, ionic strength 0.1, this peak yielded three components with mobilities ($\mu \times 10^5$) of -6.31, -5.23, and -4.49. These mobilities correspond to the mobilities of the alpha globulins.

In procedure II erythropoietic activity was almost entirely confined to fraction G. The minimal response induced by fraction F suggests that it is contaminated by a small amount of the active fraction. From the character of the effluent diagram, it appears that the material in fraction G is homogeneous. On paper electrophoresis in veronal buffer pH 8.6, ionic strength 0.075, it migrates as a

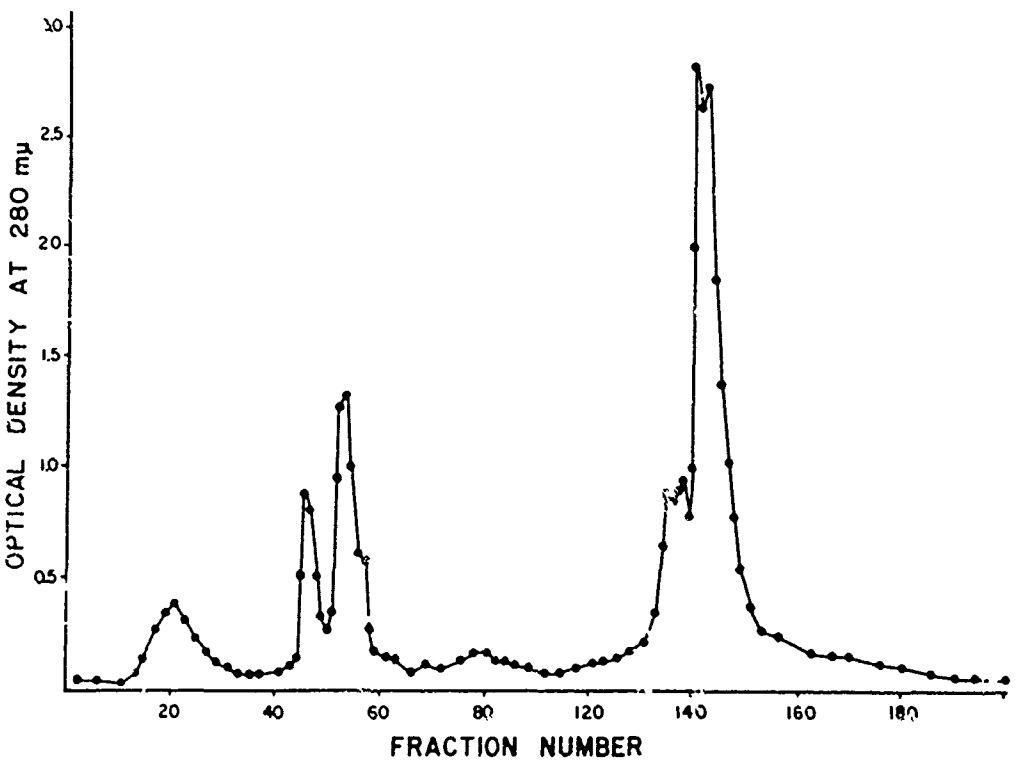

FIGURE 1

Effluent diagram, procedure I. APF (500 mg.) dissolved in 30 ml. of 0.01 molar sodium acetate buffer, pH 4.8. Solution clarified by centrifugation and supernate passed through a DEAE-cellulose column (40 × 2.5 cm.) which had been partially equilibrated to pH 6.1 with 0.01 molar acetate buffer, pH 4.8. Column washed with buffer and wash collected in fractions 1 to 77. Gradient started at fraction 77 by continuous introduction of 0.01 molar acetate buffer, pH 4.8 plus 0.25 molar NaCl into a constant volume reservoir of 200 ml. of 0.01 molar acetate buffer. Column operated at constant temperature of 25° C. Flow rate 0.65 ml./min. and fraction volume 6.5 ml. Fraction pool 44 to 56 (fraction A), 132 to 138 (fraction B), and 142 to 150 (fraction C).

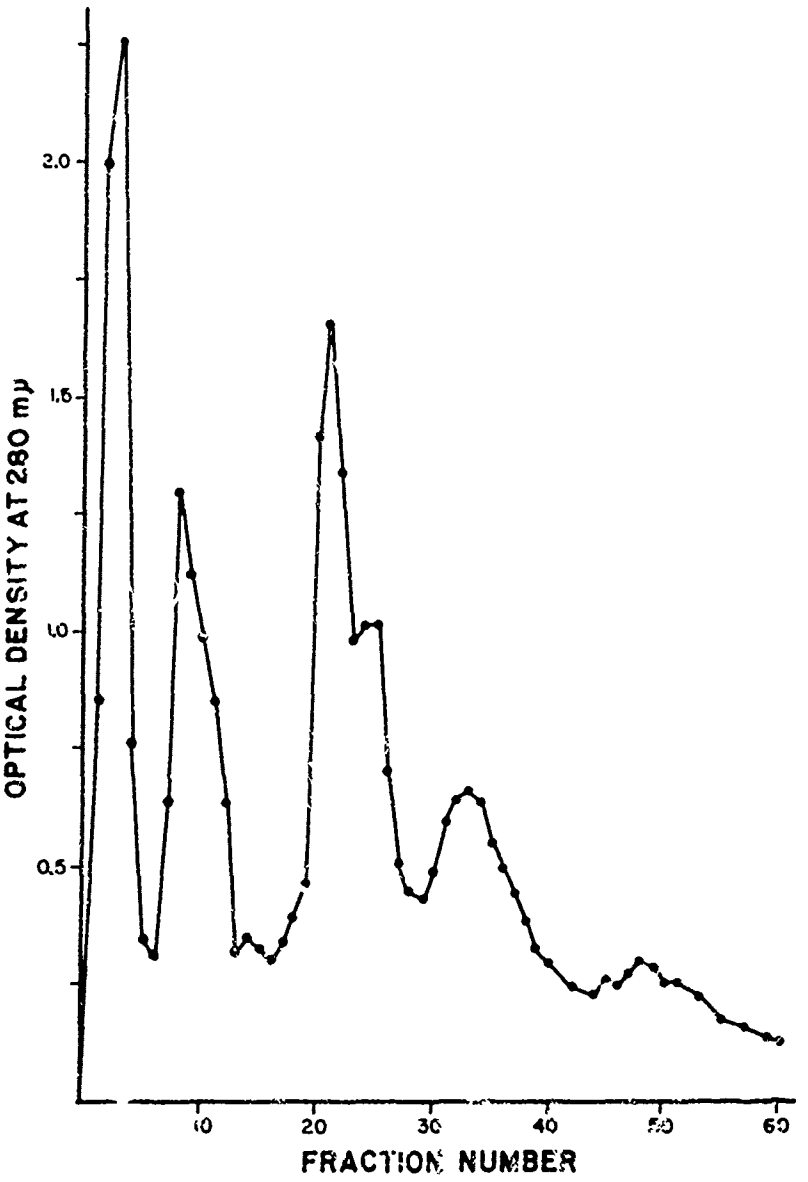

FIGURE 2

Effluent diagram, procedure II. APF (300 mg.) dissolved in 20 ml. of 0.0. molar sodium acetate buffer, pH 4.8. Solution clarified by centrifugation and supernate passed through a DEAE-cellulose column (30 × 1 cm.) which had been partially equilibrated to a pH 6.0 with 0.01 molar acetate buffer, pH 4.8. Column washed with buffer and wash collected in fractions 1 to 13. Gradient started at fraction 13 by continuous introduction of 0.01 molar acetate buffer, pH 4.8 plus 0.25 molar NaCl into a constant volume reservoir of 250 ml. 0.01 molar acetate buffer. Column operated at constant temperature of 5° C. Flow rate 0.08 ml./min. Fraction volume: fractions 1 to 13, 6.5 ml.; fractions 14 to 40, 3.5 ml. Fraction pool 1 to 4 (fraction D), 7 to 12 (fraction E), 19 to 26 (fraction F), and 30 to 40 (fraction G).

TABLE I

Erythropoietic activity of effluent fractions

Procedure I

Fraction pool	Reticulocytes *	Fe^{59} incorporation* in RBC
	(percent)	
A	2.1 ± 0.4 (4)	26.7 ± 4.3 (7)
B	1.6 ± 0.5 (4)	27.4 ± 10.4 (4)
C	6.1 ± 1.5 (4)	50.5 ± 2.2 (9)
APF	3.4 ± 0.6 (4)	39.9 ± 4.4 (4)
Saline control	1.8 ± 0.3 (4)	21.0 ± 3.6 (7)

*Mean ± S.D.

Figures in parentheses indicate number of animals in group. Values in boldface type are statistically significant, P = 0.01 or less.

TABLE II

Erythropoietic activity of effluent fractions

Procedure II

Fraction pool	Reticulocytes *	Fe^{59} incorporation* in RBC
	(percent)	
D	2.7 ± 0.6 (4)	28.3 ± 6.6 (4)
E	2.5 ± 0.4 (4)	26.9 ± 4.5 (4)
F	2.6 ± 0.4 (4)	29.6 ± 1.0 (4)
G	6.5 ± 1.3 (4)	47.6 ± 2.5 (4)
APF	3.5 ± 0.5 (4)	40.7 ± 6.9 (4)
NaCl-acetate control	2.3 ± 0.4 (4)	21.0 ± 2.6 (4)

*Mean ± S.D.

Figures in parentheses indicate number of animals in group. Values in boldface type are statistically significant, P = 0.01 or less.

single component with a mobility between that of alpha-2 and alpha-1 globulin. It stains as a glycoprotein. From its behavior on the DEAE-cellulose column it has a low isoelectric point. Its small molecular size is indicated by the failure to form a sediment when APF is centrifuged at 103,000 g for 24 hours. Its protein content as estimated by the method of Warburg and Christian is 69.3 percent. Its sialic acid content, as determined by Winzler's modification of the method of Ayala et al. (9), is 15.6 percent. Its hexose content, shown by the anthrone reaction, is 7.7 percent as glucose equivalent, and its glucosamine content, determined by a modification of the method of Elson and Morgan (10), is 10 percent. Preliminary studies suggest that the glucose content is low, and that at least a part of the hexose is present as galactose. The sialic acid content of the material is relatively high when compared with that reported for other serum glycoproteins (9), and is probably responsible for its low isoelectric point. Fraction F contains 9 percent sialic acid, while fractions D and E contain none.

Fraction G was obtained in a yield of 16 mg. Calculations based on the amount of the starting material indicate that the active fraction stimulates erythropoiesis when administered by injection of 50 μg. quantities. Preliminary dose response data using fraction G suggest that the injection of 10 μg. per day will induce a measurable erythropoietic response.

This separation and isolation of erythropoietin is reproducible, and similar results have been obtained from different lots of starting APF material. Studies are currently underway to further characterize this erythropoietic factor.

SUMMARY

A method is given for the purification of erythropoietin from the filtrate of acidified, boiled plasma prepared from phenylhydrazine anemic rabbits utilizing DEAE-cellulose ion-exchange columns. The active erythropoietic

factor thus prepared has been partially characterized and shown to be an acidic glycoprotein of low molecular weight.

The authors thank Miss Stella Gunakis, for her excellent technical assistance, and Dr. V. Koenig, for the electrophoretic and ultracentrifugal determinations.

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